RESEARCH ARTICLE

CD4+, IL17 and Foxp3 Expression in Different pTNM Stages of Operable Non-small Cell Lung Cancer and Effects on Disease Prognosis

Guo-Qing Zhang*, Feng Han, Xin-Zhi Fang, Xiao-Mei Ma

Abstract

    <u>Objective</u>: To investigate the effects of CD4+, IL17 and Foxp3 expression on prognosis of operable non-small cell lung cancer (NSCLC) with different pTNM stages. <u>Methods</u>: Expression of CD4+, IL17 and Foxp3 in 102 cases of NSCLC tissues and adjacent cancer tissues was detected by immunohistochemistry and associations with prognosis with different pTNM stages were analyzed. The Chi-square test was used to compare count data. Survival differences were evaluated by Kaplan-Meier single factor analysis and the COX regression model was used to analyze the relationship between influential factors and the disease prognosis. The significance level was $\alpha = 0.05$. <u>Results</u>: Expression of CD4, IL-17 and Foxp3 significantly varied in different pTNM stages of NSCLC tissues ($P < 0.05$). The same was true for CD4 expression ($P < 0.05$). The median survival time (MST) in the positive CD4 expression group was evidently higher than that in the negative group (25.8/23.9 months). Compared with stage III, the MST difference of stages I and II in the positive CD4 expression group were statistically significant ($P < 0.05$). The MST in positive IL-17 and Foxp3 expression groups was obviously lower than that in the corresponding negative group ($P < 0.05$) (25.6/35.1 months and 24/35.3 months, respectively). There was a significant difference of MST between any two of three stages of positive IL-17 expression group ($P < 0.05$), and it was the same with positive Foxp3 expression group. TNM stage, negative CD4 expression, and positive IL-17 and Foxp3 expression were the main risk factors for the prognosis of NSCLC. <u>Conclusions</u>: Surgical prognosis of NSCLC can be better assessed by the combination of clinical staging and expression of IL17 and Foxp3.

    Keywords: Non-small cell lung cancer - tumor immunity - prognosis - pTNM staging

Introduction

    Non-small cell lung cancer (NSCLC) is the main common type in all lung cancer cases, accounting for about 80% (Feng et al., 2010).Because there are limited effective methods for early diagnosis and its poor prognosis, the morbidity and mortality of NSCLC is shooting up in last 30 years. Therefore, it is of great importance to explore the underlying molecular immune mechanisms and suitable markers associated with the lung cancer progression (Tian et al., 2011).The immune function of patients with NSCLC is closely related with the occurrence, development and prognosis of tumor. Understanding the causes of immunosuppression in NSCLC patients is clinically significant for assisting patients to reconstruct immune balance, enhance anti-tumor capability and reduce cancer recurrence and metastasis.

    CD4+T cells are significantly important in removing cancerous tissue or cells. The initial CD4+T cells can be activated together by antigen costimulatory signals and costimulatory molecules, and be differentiated into different subtypes, including helper T cells (Th1, Th2 and Th17) and regulatory T cell (Treg). They perform different biological functions in anti-tumor immunity, tumor immune evasion; play an important role respectively in tumor tolerance mechanisms, tumor immune microenvironment and immune homeostasis (Sallusto et al., 2009). Recent researches show that, CD4+T cells can directly kill tumor cells (Thomas et al., 2008). There are a lot of intermediate states of CD4+T cells. Among them the induced Foxp3+T cells (iTreg) and Th17 cells could be mutually transformed, but their roles in tumor immunization remain to be clarified. Th17 cells can secrete interleukin 17 (IL-17) as a primary effector. It can mediate inflammatory response, participate in occurrence and development of autoimmune diseases and tumor, promote tumor growth and angiogenesis, and determine prognosis of disease (Dong et al., 2008; Kornt et al., 2009; Veldhoen et al., 2009). Treg cells are a class of T cell subsets with immunosuppressive effect, and mainly

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are CD4⁺CD25⁺Treg cells. Owing to their low reactivity and immune suppression function, Treg cells can block the antitumor immunity to obtain immune escape (Huang et al., 2009) and promote the occurrence and development of tumor (Beyer et al., 2009; Liu et al., 2009). Foxp3 protein is the specific marker of Treg cells (Wan et al., 2007; Williams et al., 2007). The Foxp3 gene plays an important role in the regulation of Treg cell development. There is no Treg cell in the rat with Foxp3 knockout. The investigation of Foxp3 protein expression in tumor tissues contributes to the understanding of inhibitory effect of CD4⁺CD25⁺Treg cells in tumor microenvironment (Woo et al., 2001; Gray et al., 2003).

As the expression of Th17 cells in tumors and the related action mechanisms are not clear until now, the recognition of Th17 cells on influencing the function of NK cells and effector T cells, and inhibiting the tumor immune molecule activity are also limited. Whether Th17 cells promote or inhibit the tumor development in tumor microenvironment is not completely determinated, especially on their relationship with tumor prognosis. Therefore, the functions of Th17 cells, and the biological characteristics of their effect factors in tumor microenvironment are to be further confirmed.

In this study, the expression of CD4⁺, IL17 and Foxp3 in 102 cases of NSCLC tissues and adjacent cancer tissues was detected by immunohistochemistry method. Their effects on invasion and progression of NSCLC, and on pathological characteristics and prognosis were discussed. The objective is to provide a research foundation for targeted biological treatment of NSCLC and monitoring of its occurrence, development and prognosis.

Materials and Methods

General materials

102 cases of NSCLC tissue samples were collected from patients (66 males and 36 females) in Affiliated Tumor Hospital of Xinjiang Medical University, China between January 2007 and December 2009. The NSCLC tissues samples were completely excised and confirmed by pathology. The adjacent cancer tissues were excised 3cm from the NSCLC tissues. All patients were not treated with radiotherapy or chemotherapy before, and their age was 40-73 years with mean age, 65 years. All samples were divided into 44 cases of squamous cell carcinoma, 53 cases of adenocarcinoma and 5 cases of adenosquamous carcinoma, referring to the "Rosai and Ackerman's surgical pathology (10th) ". They were pathologically staged as stageI (42 cases), stage II (27 cases) and stage III (33 cases), according to the TNM classification criteria for lung cancer formulated by the International Union Against Cancer (IUAC) in 2007. General data were shown in Table 1.

Reagents and Methods

Reagents: Concentrated mouse anti-human IL-17A antibody and Foxp3 antibody was purchased from Wuhan Boster Biological Engineering Co., Ltd and Abcam (Hong Kong) Limited, China, respectively. EliVision two-step kit and concentrated mouse anti-human CD4 antibody

were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd, China.

Method: All samples were stained using immunohistochemical Elivision method, after high-temperature antigen retrieval. After DAB coloration, recoloration, dehydration, sealing, dewaxing and hydration, the samples were diagnosed and pathologically staged by physicians in the pathology department. The standard CD4 and IL17A were used as positive control, and PBS was used as negative control.

Determination of results: Brown granules in cytoplasm/membrane, cytoplasm and nucleus of T cells represent the positive expression of CD4, IL-17 and Foxp3, respectively. According to the intensity of staining, the expression levels were divided as follows: (-) level (There was no brown granule or the color of coloring area was uniformly pale yellow, the same with the background), (+) level (There was a few visible light yellow granules and the color of coloring area was obviously deeper than background, or there were more dark brown granules in cytoplasm) and (++) level (There were many dark brown granules in coloring area. (Figure 1)

Follow-up visits

Follow-up visits were conducted from the operation date to December 31, 2011. In 102 cases of patients, 8 cases were lost to follow-up. The follow-up rate was 92.2% with the median follow-up time, 30.2 months.
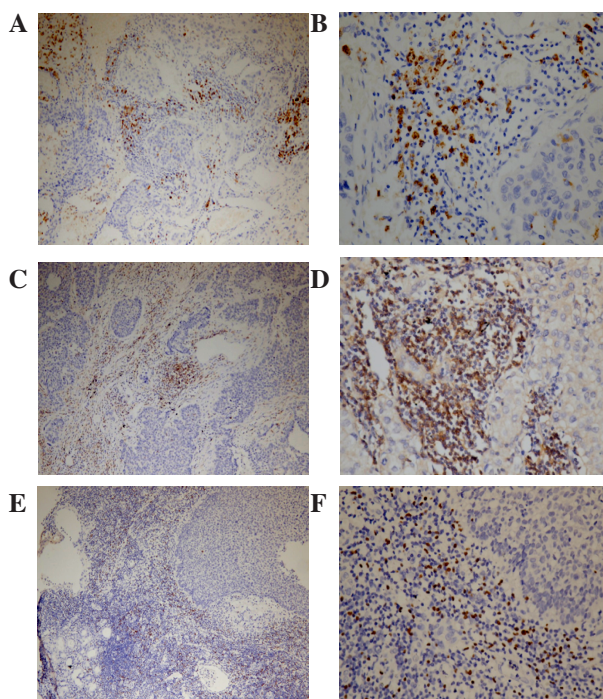

Figure 1. (A) Positive IL-17 Expression in NSCLC Tissues (Magnification: 10×10); (B) IL-17 Expression in Cytoplasm of NSCLC Tissues (Magnification: 40×10); (C) CD4 Expression in NSCLC Tissues (Magnification: 10×10); (D) CD4 Expression in Cytoplasm/Membrane of T Cells (Magnification: 40×10); (E) Positive Foxp3 Expression in NSCLC Tissues (Magnification: 10×10); (F) Foxp3 Positive Expression of in Nucleus of T Cells (Magnification: 10×10)

Table 1. Relationship Between CD4, IL-17 and FoxP3 Expression and Clinical Factors in NSCLC

Clinical factors	IL17 expression								Foxp3 expression							CD4+ expression						
	NSCLC tissues				Adjacent cancer tissues				NSCLC tissues				Adjacent cancer tissues			NSCLC tissues				Adjacent cancer tissues		
	-	+	++	P value	-	+	++	P value	-	+	++	P value	-	+	P value	-	+	++	P value	-	+	P value
Sex																						
Male	21	25	20	0.9	21	38	7	0.454	11	41	14	0.346	51	15	0.386	12	35	19	0.847	29	37	0.165
Female	10	15	11		11	18	7		8	17	11		25	11		7	17	12		21	15	
Age																						
≤45	3	3	2	0.783	1	7	0	0.729	1	6	1	0.761	7	1	0.965	0	7	1	0.326	0	0	0.961
>45, <60	6	17	9		8	23	1		5	23	4		27	5		6	16	10		25	17	
≥60	18	28	16		20	40	2		10	38	14		52	10		13	30	19		36	24	
Pathological type																						
Squamous cell carcinoma	12	23	14	0.862	13	33	3	0.184	4	34	11	0.114	37	12	0.996	7	27	15	0.554	25	24	0.229
Adenocarcinoma	15	25	13		16	37	0		12	33	8		40	13		12	26	15		34	19	
pTNM staging																						
StageI	19	14	9	0.008*	12	30	0	0.616	12	22	8	0.024*	37	5	0.258	6	21	15	0.002*	19	23	0.314
StageII	6	14	7		7	19	1		2	16	9		24	3		3	16	8		22	15	
StageIII	5	11	17		10	21	2		2	17	14		25	8		15	16	2		20	13	
Tissue differentiation																						
High differentiation	0	4	0	0.281	2	1	1	0.863	3	1	1	0.078	3	3	0.117	1	2	1	0.997	11	10	0.697
Medium differentiation	15	24	16		14	27	14		7	34	13		43	11		11	27	17		25	16	
Low differentiation	13	19	11		12	20	11		9	28	6		36	6		9	22	12		21	19	

*P<0.05 was considered as statistically significant

Table 2. Results of COX Regression Analysis on Influential Factors for NSCLC Patients

	B	SE	Wald	P	OR	95% confidence interval
Age	0.021	0.019	0.121	0.285	1.016	0.975-1.163
Pathological type	0.319	0.287	1.867	0.154	1.219	0.845-2.726
TNM staging	0.296	0.578	5.899	0.019	1.474	0.246-2.309
CD4 expression	1.258	0.429	3.994	0.046	1.359	1.017-5.473
IL-17 expression	1.314	0.162	3.029	0.023	1.387	1.013-1.828
Foxp3 expression	1.157	0.388	5.262	0.014	1.48	1.476-5.680

The loss of follow-up was calculated according the last follow-up time. The period from diagnose date to death or deadline time was regarded as the survival time. Completely lost cases and non-tumor death cases were treated according to the censored data processing method in statistical analysis.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software. A Chi-square test was used to compare count data. Survival differences were evaluated by single factor analysis using Kaplan-Meier method. The COX regression model was used to analyze the relationship between influential factors and prognosis of disease. The significance level was $\alpha = 0.05$.

Results

Relationship between CD4, IL-17 and Foxp3 expression and clinical factors in NSCLC

As shown in Table 1, there was a statistically difference of the expression of CD4, IL-17 and Foxp3 in different pTNM stages of NSCLC tissues (P < 0.05), respectively. In NSCLC tissues, the expression of CD4, IL-17 and Foxp3 in patient with different age, sex, tumor pathological type and tumor tissue differentiation degree were not statistically different (P > 0.05), respectively, the same with those in adjacent cancer tissues. In addition, the CD4, IL-17 and Foxp3 expression in different pTNM stages of adjacent cancer tissues were not significantly different (P > 0.05), respectively.
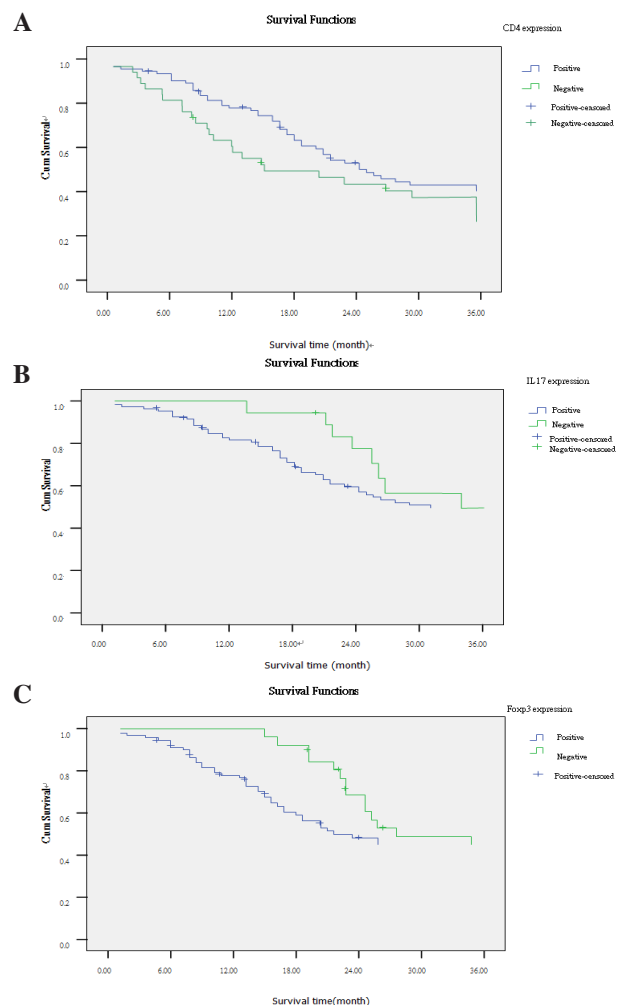
Relationship between CD4, IL-17 and Foxp3 expression

Figure 2. Comparison of Survival Time in Positive and Negative (A) CD4 Expression Groups; (B) IL-17 Expression Groups; (C) Foxp3 Expression Groups

and prognosis of NSCLC

As shown in Figure 2, in 102 cases of NSCLC tissues, the median survival time (MST) in the positive CD4 expression group was significantly higher than that in the negative group (P < 0.05) (25.8/23.9 months). The MST in positive IL-17 and Foxp3 expression group was obviously lower than that in the corresponding negative group (P < 0.05) (25.6/35.1 months and 24/35.3 months,
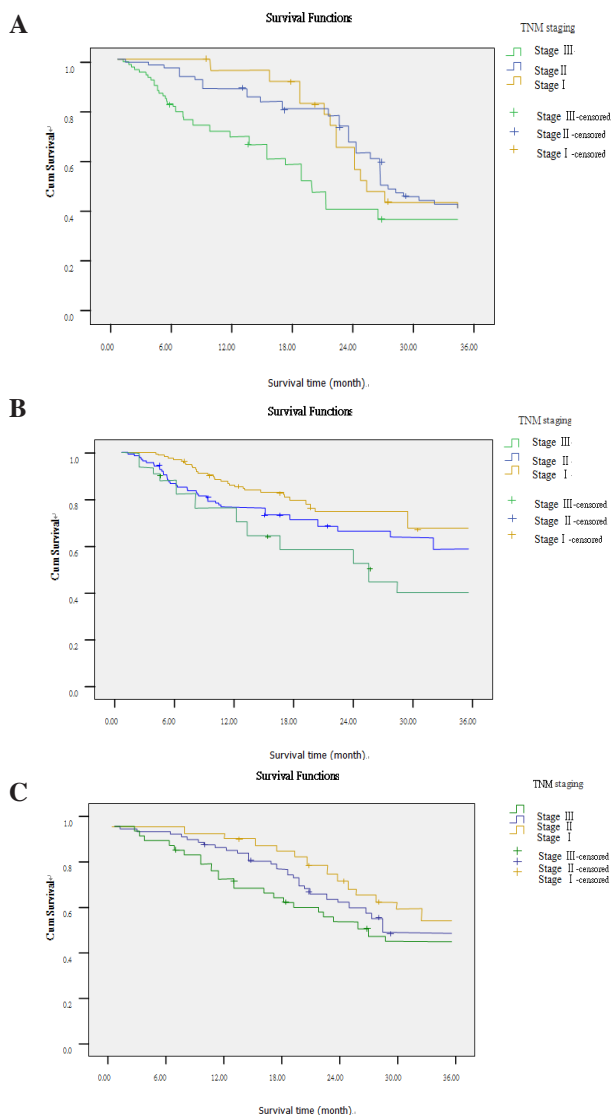
A

B

C

Figure 3. Relationship Between Positive (A) CD4; (B) IL-17; (C) Foxp3 Expression and Prognosis in Different TNM Stages of NSCLC

respectively).

Effects of CD4, IL17 and Foxp3 expression on prognosis of NSCLC with different pTNM stages

Effects of CD4, IL17 and Foxp3 expression on prognosis of NSCLC with different pTNM stages were shown in Figure 3. There was no significant difference of MST between stageI and stageII of positive CD4 expression group (P > 0.05). While compared with stage III, the MST difference of stageIand stageIIwere statistically significant (P < 0.05), respectively. There was an evident difference of MST between any two of three stages of positive IL-17 expression group (P < 0.05), the same with the positive Foxp3 expression group.

Results of Cox regression analysis

Results of Cox regression analysis were shown in Table 2. The age, sex and tumor pathological type of NSCLC patients were not correlated with the prognosis of NSCLC. The TNM staging, negative CD4 expression, and positive IL-17 and Foxp3 expression were the main risk factors for prognosis of NSCLC.

Discussion

The immune function reduction and tumor metastatic recurrence are the main causes of treatment failure of NSCLC. Cellular immunity is an important part of the immune system, and plays a major role in removing cancerous tissues and preventing intrusion of pathogenic bacteria. T cells occupy a central position in the cellular immunity (Winter et al., 2007). Early researchers believe that the majority of tumors only express MHC class I antigen, and not for MHC class II antigen. This characteristic enables the alien cells to be recognized only by CD8+T cells, and not by CD4+T cells. However, it is found that CD4+T cells can reversely induce antigen-presenting cells (APC) to supply CD8+T cells and other immune cell costimulatory molecules (Greenberg et al., 1985). Though there is only low level of MHC class II antigen express in entitative tumor cells, the IFN γ stimulation can upregulate the expression of MHC class II antigen. So CD4+T cells can directly kill tumor cells. The MHC class II antigen is mainly expressed in APC, and is involved in the exogenous pathway of antigen presentation (endosomal/lysosomal pathway). The T cell receptor (TCR) in CD4+ T cell surface is activated by MHC class II antigen, and then the immune response is initiated. In addition, the low expression of CD4 can lead to the decrease of helper T cells and reduction of body's immune function. Thus, the tumor occurs, develops and metastasizes. In recent years, researchers (Hunder, et al, 2008) have compared the killing effect on HY antigen expressing tumors between CD8+T cells and CD4+T cells. They find that CD4+T cells have stronger killing effect than CD8+T cells. A recent research shows that, as the tumor associated antigen (TAA) can be directly recognized by CD4+T cells, tumor cells can be killed and cleared even in the absence of MHC class II antigen (Denardo et al., 2009).

Results of our study show that the difference of CD4 expression in different pTNM stages of NSCLC tissues are statistically significant, and the MST in positive CD4 expression group is higher than that in the negative group. It can be speculated that there is a higher expression of MHC II antigen in entitative tumors of NSCLC with high levels of CD4+T cells. CD4+T cells have a stronger ability to directly or indirectly kill tumor cells and prolong the survival time of patients.

In this study, there was no significant difference of MST between stageI and stageII of positive CD4 expression group, but the differences between stageI and stage III, stage II and III stage were significant, respectively. The reason may be that the expression level of MHC II antigen in early entitative tumor cells is higher, which results in the recognition and killing of tumor cells by CD4+T cells. Meanwhile, it is suggested that the current preoperative TNM staging is imperfect, and the accuracy of preoperative examination and the systemic cleaning on lung hilum and mediastinal lymph node need to further strengthened.

CD4+T cells can be activated by TGF-β and transformed into Treg cells. Foxp3 protein, the specific markers of Treg cells, can be expressed in tissues of NSCLC, ovarian,

melanoma, gastrointestinal tract malignancy, pancreatic malignancy and their peripheral blood. Along with the occurrence and development of tumor, the amount of Foxp3 protein increases gradually, while the amount of locally infiltrated $CD8^+TIA^+CTL$ decreases gradually and even disappears. Thus, the immunosuppression is produced. This suggests that high expression of Treg cells can promote the development of tumor, and affect the prognosis for patients (Woo et al., 2001; Gray et al., 2003; Kono et al., 2006; Hiraoka et al., 2006; Kobayashi et al., 2007).

Thl7 cell is a new subsets type of $CD4^+T$ cell. It can stimulate the immune response, clear the pathogens and cancerous cells or tissues, and participate in the inflammation reaction (Park et al., 2008). Studies (Zhang et al., 2008) find that, the percentage of IL-17 cells in peripheral blood of gastric cancer patients increases, compared with normal patients. The increase degree is related to clinical staging of gastric cancer. Compared to the mesenteric lymph node, the levels of Th17 cells in the draining lymph node of the patients with advanced gastric cancer (II, III and IV stages) are elevated. The mRNA expression levels of Th17 cell related factor IL-17, IL-23p19 and RORc significantly increase, the same to the IL-17 and IL-23 concentration in serum. So it is believed that Th17 cells may promote the pathological development of gastric cancer. Another studies (Le et al., 2008; Zhang et al., 2009) find that the proportion of Th17 cells in colon and hepatocellular carcinoma tissues is significantly higher than that in normal tissues, respectively. Larmonier et al. (2007) find that, both level of Th17 cells and level of Treg cells in gastric cancer patient have a growing trend, but it is more obvious for Treg cells. This suggests that in tumor microenvironment, Thl7 cells and Foxp3+Treg cells have a synergistic effect, together promoting the occurrence and development of tumor.

In this study, the MST in positive IL-17 and Foxp3 expression group is lower than that in the corresponding negative group, respectively. There is a significant difference of MST between any two of three stages of positive IL-17 expression group, the same with positive Foxp3 expression group. This indicates that Thl7 cells and Foxp3+Treg cells can together promote the occurrence and development of tumor. Results of Cox regression analysis show that, the TNM staging, negative CD4 expression and positive IL-17 and Foxp3 expression are the main risk factors for prognosis of NSCLC. The negative CD4 expression and high IL-17 and Foxp3 expression could promote the invasion and metastasis of NSCLC. During the differentiation process of CD4 cells to Th17 and Foxp3+Treg cells, low level expression of tumor related antigen is improved and the immune suppression and escape are formed. Meanwhile, Thl7 cells and Foxp3+Treg cells interact with each other to cause the myeloid-derived suppressor cells (MDSC) to move toward the inflammatory environment, which promotes the growth of tumor. These may be related to the complex etiology of tumor.

In conclusions, the Treg cell-mediated immune tolerance is closely related to the occurrence, development and prognosis of NSCLC. The prognosis of NSCLC can be better evaluated by the combination of clinical staging and expression of IL17 and Foxp3 in NSCLC tissues. The operational resection of tumor can reduce the in vivo tumor environment and amount of Treg cells, and relieve the immune suppression. This is a foundation of the immune function restoration for postoperative patients. Detection of expression of IL17 and Foxp3 in NSCLC tissues can help to understand the local immune response in tumor microenvironment, and provide a basis for postoperative antitumor immunity. This contributes to better understanding the mechanism of tumor immune tolerance and improving the therapeutic level of NSCLC.

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